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13. ABSTRACT (Maximum 200 Words)

Our proposal aims to investigate in details the potential function of CHK in breast cancer as a signal transducer in the signaling pathway from HER2/Neu receptor. The studies conducted in the period reported included: 1) studies on the effects of CHK on the HER2/Neu-induced tumorigenesis in vivo, and 2) generation of CHK-derivatives. Our major findings are as follows: Task 2: We created transgenic mice to study the effects of CHK on ErbB2-mediated signaling in vivo as well as on ErbB2-induced mammary tumor formation in vivo. Task 3: We used an alternative approach to successfully generate CHK derivatives which can be used in experimental CHK-based protein therapy of mammary tumors.

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Title: **EFFECTS OF CSK HOMOLOGOUS KINASE OVEREXPRESSION ON HER2/NEU-MEDIATED SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCER CELLS.**

P.I.: RADOSLAW ZAGOZDZON, M.D., PH.D.

INTRODUCTION: The onset of 90% of all breast cancers is random and spontaneous, while 10% of cancers have been linked to specific mutations in autosomal dominant breast cancer susceptibility genes such as BRCA1 and BRCA2. Random onset of breast cancers has in many cases been correlated with increased HER2/Neu (also termed ErbB-2) expression and Src family tyrosine kinases activity. The exact molecular mechanism of this phenomenon remains unknown. The initial steps in HER2/Neu pathways are complex, and are modulated by such processes as the autophosphorylation, cross-phosphorylation and dimerization of ErbB molecules. Furthermore ErbB cascades also interact with other signaling pathways. Association of c-Src with these receptor tyrosine kinases (PTKs) is an integral part of the signaling events mediated by the receptors, and may contribute to the malignant transformation of cells. Increased Src kinase activity observed in HER2/Neu-induced tumors results from the ability of the Src-SH2 domain to directly interact with HER2/Neu in a tyrosine phosphorylation-dependent manner. Since HER2/Neu and pp60src play a role in breast cancer and are altered during malignant transformation and tumor progression, it is important to characterize the regulation of these protein kinase activities, and the likely interactions of these kinases with each other.

Src family kinase activity is inhibited by the phosphorylation of a conserved, carboxy-terminal tyrosine. The protein tyrosine kinase responsible for this phosphorylation is Csk. We and others identified a second member of the Csk family – Csk Homologous Kinase (CHK). CHK has been suggested to have a specific role in breast cancer and the potential to be a target of breast cancer drug development. CHK, which is specifically expressed in primary breast cancer specimens, but not in normal breast tissues, phosphorylates Src and down-regulates its activity. Previous biochemical data also suggested that CHK acts as a negative growth regulator of human breast cancer. Furthermore, the interaction between the CHK-SH2 domain and $pTyr^{1248}$ of the HER2/Neu receptor is specific and critical for CHK function.

BODY:
The results mentioned above lead us to hypothesize that: (i) CHK is able to antagonize the growth-promoting signals that are mediated by HER2/Neu and Src kinases; (ii) enhancement of the binding affinity of CHK to the HER2/Neu protein might further increase the antitumor effects of CHK in breast cancer cells; (iii) peptide derived from an enhanced-binding mutant of CHK may retain its inhibiting ability on HER2/Neu and Src-mediated signaling. Therefore, the goals of this project are: (1) to investigate the effects of CHK on downstream signaling from the HER2/Neu receptor; (2) to assess the possibility of enhancing the inhibitory effects of CHK on HER2/Neu-mediated signaling; (3) to test the anti-tumor effects of CHK-derived peptides designed to diminish the transformation potential of HER2/Neu.

Task 2

To study the effects of CHK on ErbB2-induced tumorigenesis in breast in vivo, we initially proposed injections of the stable CHK-transfected breast cancer cell lines into nude mice. While these experiments are still part of our experimental design, they deal with already established in vitro cell lines with little correlation to the natural stages of mammary carcinogenesis occurring in a live organism. Therefore, we also decided to employ an alternative and more nature-related model of mammary tumorigenesis in vivo, namely the mammary-tissue specific transgenic model.

Generation of CHK transgenic mice. We have generated an MMTV-CHK-SV40 construct. The HindIII restriction site within the CHK gene was deleted (without altering the amino acid sequence) using the PCR reaction. CHK was then cloned between the HindIII and EcoRI restriction sites of the MMTV-SV40-BSSK+ plasmid (generous gift from Dr. Philip Leder). Then, the MMTV-SV40-BSSK construct with the CHK insert was linearized with SalI and SpeI restriction enzymes. The CHK DNA was microinjected into pronuclear stage zygotes. To identify transgenic progeny, genomic DNA was extracted from 1.0-cm tail clippings by digestion with proteinase K (Roche), followed by phenol/chloroform extraction. The nucleic acid pellet was resuspended in 350 µl of distilled water, 2 µl of the DNA solution was then subjected to 35 cycles of PCR with transgene specific primers, followed by electrophoresis on a 1% agarose gel and staining with propidium iodide (Fig. 1A). Transgene positive animals were further analyzed by Southern blotting. 15 µl of the DNA solution was then digested with 100 U of EcoRI and SpeI overnight followed by gel electrophoresis and Southern blot transfer to Hybond N (Amersham) membrane. The membrane was hybridized with a transgene specific radiolabeled ([γ-^{32}P]dCTP) probe of full-length CHK cDNA (Figure 1B). To analyze the germ line transmission, pups derived from transgenic progeny (F_0) and wild-type FVB/N animals were screened for germ line transmission of the MMTV-CHK transgene. To date, we have obtained three germ line transmissions (Fig. 1C). All of the strains have expression of transgenic CHK on mammary tissue (Fig. 2A) and in all of these three strains, pp60^{src} kinase activity is diminished (Fig. 2B).

Development of mammary glands in MMTV-CHK transgenic animals: The CHK transgene is expressed in several different tissues (Fig. 3), such as spleen. We next examined the development of mammary glands (whole mount staining). As shown in Fig. 4 we did not observe any changes in the developing mammary glands in CHK transgenic mice.

Interestingly, we found that the mouse form of CHK (Ctk) is expressed in mammary glands (MG) as well as in the tumors formed in these transgenic animals that are prone to develop mammary tumors (MMTV-Neu, MMTV-NeuT, MMTV-c-myc) (Fig. 5), but not in normal mammary glands. These results are in agreement with our data on the expression of CHK in primary human breast tumors (*1*).

Generation of double transgenic mice: We are currently generating double transgenic mice, and have already made progress in generating CHK/c-Myc and CHK/Neu double transgenic mice as shown in Fig. 6.

Task 3

"Protein therapy" is a newly developed method which allows proteins, peptides and biologically active compounds to penetrate across the plasma membrane of eukaryotic cells via homopolymers or peptides containing a high percentage of cationic amino acid protein transduction domains (such as polyarginine, 9-12-arginine peptide tag) (*2, 3*). This method enables us to control the localization of targeted substances in subcellular compartments, such as the nuclei, mitochondria and post-synaptic density. The method is very efficient and applicable not only to cultured cells but also to tissue slices and the whole animal. Brain, heart, skeletal muscle, liver, pancreas and lymphocytes are efficient target organs and tissues for protein therapy (for review see (*4*)).

In our project we proposed to study the effects of CHK-derived peptides fused with polyarginine on Neu signaling in breast cancer cells. However, based on our NMR studies of SH2 domain of CHK, recently published and reported in previous annual report, we disqualified the primarily designed peptides from further studies. Our studies suggested that whole SH2 domain of CHK instead of particular peptides is necessary for this protein to interact with activated Neu (Kim S., personal communication). Thus, we chose an alternative approach, and designed full-length CHK-based polyarginine-fusion protein derivative as a research tool and potential therapeutic agent.

Generation of a CHK-based polyarginine-fusion protein derivative as a research tool and potential therapeutic agent: Introduction of polyarginine fusion proteins into mammalian cells: To initially estimate the uptake of polyarginine fused protein into T47D breast cancer cells, we introduced cDNA encoding nine consecutive arginines into pGEX4T-1 vector and then expressed and purified the GST-9Arg fusion protein in the E. coli expression system. We estimated the uptake of this protein by PC12 cells in-vitro. As shown in Fig. 7, incubation of cells for 30 min with GST-9Arg protein concentrations as low as 4 μg/ml resulted in the appearance of the specific protein band in the total cell lysate when assessed by Western blotting with anti-GST antibody. Another construct of this type created in our lab is EGFP-12Arg fusion protein. Our time point experiments show that when cells are incubated in the presence of GST-9Arg or EGFP-12Arg fusion proteins, each of these proteins can be detected within the cells without apparent toxicity continuously for at least 24 or 72 hours, respectively (data not shown).

Generation of expression vectors encoding CHK-polyarginine fusion protein: Recently, transduction of an active enzyme, human catalase, mediated by arginine-rich peptides into mammalian cells, was reported (*5*). Based on these results, we attempted to generate a CHK-polyarginine derivative as a potential tool to study the role of CHK in breast cancer. Our first choice was to express full-length CHK-polyArg fusion protein in the bacterial expression system. Unfortunately, our experiments indicate that CHK expression in E. coli undergoes excessive proteolitic degradation within the kinase domain, which results in dramatically little yield (data not shown). Our observations were confirmed by a recent publication by Ayarpetov et al. (*6*). Therefore, we chose a mammalian expression system instead to produce and subsequently purify CHK-polyArg protein. Additionally, we tagged the C-terminus of CHK with GFP, to enable visual control of the protein uptake and subsequent subcellular localization. Schematic maps of the generated vectors (control GFP-12Arg and CHKwt/dk-GFP-12Arg) are presented in Fig. 8A. We analyzed the expression of newly generated proteins after transient transfection of CHO cells by Western blotting using anti-CHK and anti-GFP antibodies (Fig. 8B). We also assessed the transiently transfected CHO cells under a fluorescent microscope. As shown in Fig. 9, all proteins containing 12Arg tended to densely localize in the nucleoli. This fact is not surprising, because Arg is a cationic amino acid and, when polymerized, is capable of binding anionic structures, such as nucleic acids. This capability was recently used in non-viral gene delivery techniques (for review see (*7*)). However, while GFP-12Arg protein showed a widely diffuse localization within the transfected cells (Fig. 9A), both CHKdk and CHKwt localized mostly within the cytoplasm (Fig. 9B and 9C, respectively). This strongly suggests the potential usefulness of our newly generated constructs in studies on the subcellular localization of CHK and co-localization with other proteins. Our hope is to achieve therapeutic antitumor effects by the application of CHK-polyArg derivatives.

Some disadvantage of the mammalian expression system to obtain purified proteins is a small yield, which can slow down our studies. Therefore, we attempted to create another CHK derivative, which can be expressed in bacteria. To this end, we fused SH3 and SH3 domains of CHK with kinase domain of Csk.

Construction of CHK/Csk (CHSK) chimeric fusion cDNA. To generate CHK/Csk (CHSK) chimeric fusion cDNA, an Xba I restriction site was introduced by PCR into both cDNA

sequences in the region between SH2 and kinase domains (Fig. 10).

Then, both PCR products were digested with appropriate restriction enzymes, ligated pIRES2-EGFP plasmid (Clontech) with the use of Nhe I and Xho I enzyme sites. Ligation product was evaluated by restriction enzyme analysis, sequencing, and transient tranfection of 293T followed by Western Blot (Fig. 11A). Enzymatic activity of newly generated CHSK chimeric protein was assessed using in vitro tyrosine kinase assay (Fig. 11B, C), as described previously (*1*).

For further analysis of CHSK chimeric protein we attempted to express and to purify CHK, Csk and CHSK as GST-fusion proteins in E. coli expression system. In contrast to CHK, CHSK and Csk were successfully expressed in this system retaining their kinase activities after purification (data not shown). Currently, we are in process of generating the CHSK-9Arg fusion construct, and we plan to compare the biological effects (e.g. association with the activated ErbB2 and inhibition of Src activity) of CHSK with these of wild-type form of CHK.

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1. C. Bougeret, S. Jiang, I. Keydar, H. Avraham, *J. Biol. Chem.* 276, 33711 (August 31, 2001, 2001).
2. D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman, J. B. Rothbard, *J Pept Res* 56, 318 (Nov, 2000).
3. K. Han *et al.*, *Mol Cells* 12, 267 (Oct 31, 2001).
4. H. Matsui, K. Tomizawa, Y. F. Lu, M. Matsushita, *Curr Protein Pept Sci* 4, 151 (Apr, 2003).
5. L. H. Jin *et al.*, *Free Radic Biol Med* 31, 1509 (Dec 1, 2001).
6. M. K. Ayrapetov, S. Lee, G. Sun, *Protein Expr Purif* 29, 148 (Jun, 2003).
7. M. Nakanishi *et al.*, *Curr Protein Pept Sci* 4, 141 (Apr, 2003).

KEY RESEARCH ACCOMPLISHMENTS:

Our major accomplishments are as follows:

Task 2. We created transgenic mice to study the effects of CHK on ErbB2-mediated signaling in vivo as well as on ErbB2-induced mammary tumor formation in vivo;

Task 3. We used an alternative approach to successfully generate CHK derivatives which can be used in experimental CHK-based protein therapy of mammary tumors.

REPORTABLE OUTCOMES:

So far we published 3 original papers as a result of our project (the papers have been attached to the previous annual report):

1. McShan, G.D., R. Zagozdzon, S.Y. Park, et al., *Csk homologous kinase associates with RAFTK/Pyk2 in breast cancer cells and negatively regulates its activation and breast cancer cell migration.* Int J Oncol, 2002. **21**(1): p. 197-205.
2. Zagozdzon, R., C. Bougeret, Y. Fu, et al., *Overexpression of the Csk homologous kinase facilitates phosphorylation of Akt/PKB in MCF-7 cells.* Int J Oncol, 2002. **21**(6): p. 1347-52. (Note: The article is attached in a manuscript format, as the journal format is currently unavailable to us)
3. Kim, S., R. Zagozdzon, A. Meisler, et al., *Csk homologous kinase (CHK) and ErbB-2 interactions are directly coupled with CHK negative growth regulatory function in breast cancer.* J Biol Chem, 2002. **277**(39): p. 36465-70

CONCLUSIONS:

Our recent results confirm the notion that CHK is a signal transduction modulator following activation of HER2/Neu receptor in breast cancer cells. We are continuing our studies according to the previously approved Statement of Work with additional the use of some alternative approaches, based on the recent findings in the field of breast cancer studies, as stated within this report.

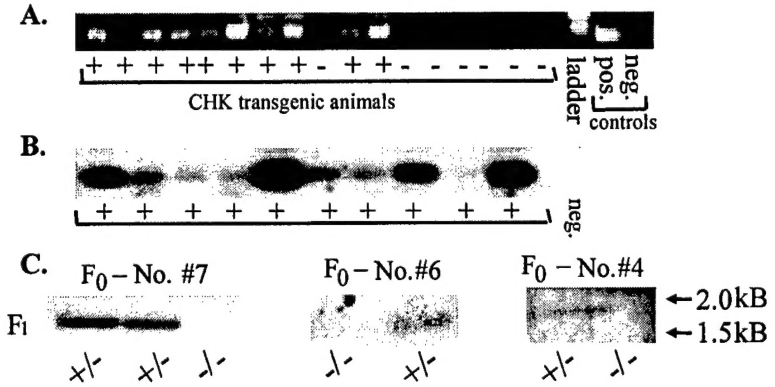

Figure 1. A. Initial screen for potential founders (F_0). **B.** Southern blot confirmation of transgene integration in F_0. **C.** Southern blot of litters (TgN x wt). Genotypes of animals are shown under the blot

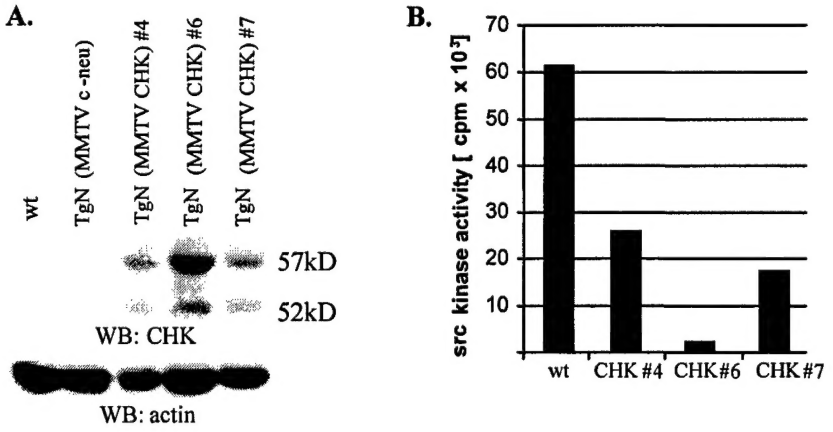

Figure 2: A. Expression of CHK in the mammary glands of transgenic mice and wild type (wt) mice. Total cell lysates were prepared from mammary glands and 50 µl of each sample was analyzed by Western blot analyses. **B.** Activity of $pp60^{src}$ in the mammary glands of CHK transgenic animals.

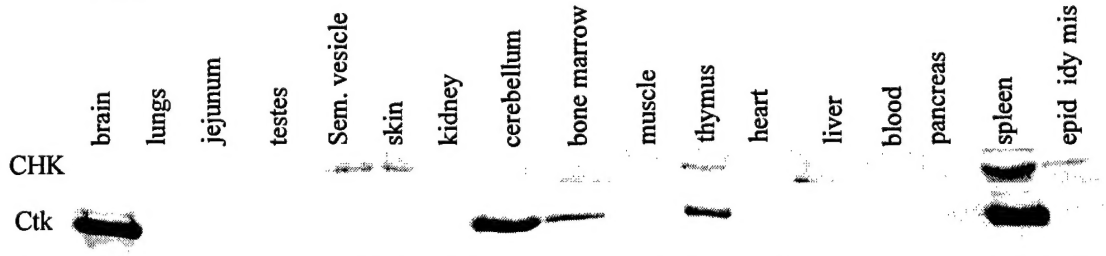

Figure 3: Expression of transgene (human CHK) and Ctk (mouse form of CHK) in different organs in MMTV-CHK transgenic mice. Total cell lysates from these tissues were prepared and analyzed by Western blotting.

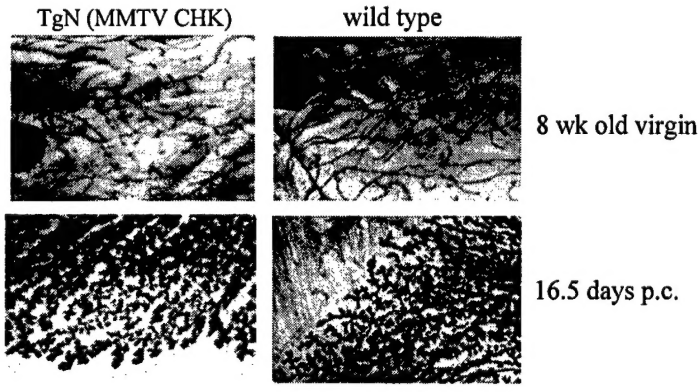

Figure 4: Whole mount staining of mammary glands of CHK transgenic mouse (TgN) vs. wild type (wt). Development of mammary glands in CHK transgenic animals is similar to that observed in wt mice. Branching of mammary ducts as well as terminal buds is well developed in the control and CHK transgenic mouse.

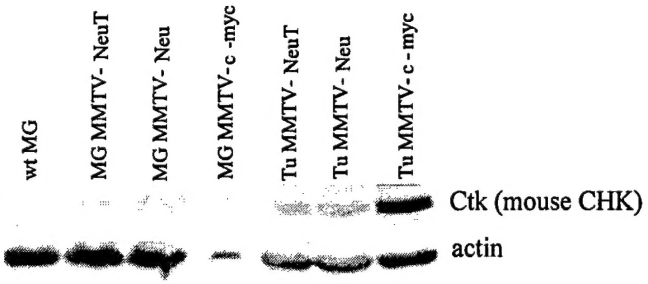

Figure 5: Expression of Ctk (mouse form of CHK) in the mammary glands of mice prone to develop mammary tumors (MMTV-Neu, MMTV-NeuT, MMTV-c-myc), and in mammary tumors developed in these mice (MG-mammary gland tissue lysate, Tu – mammary tumor lysate).

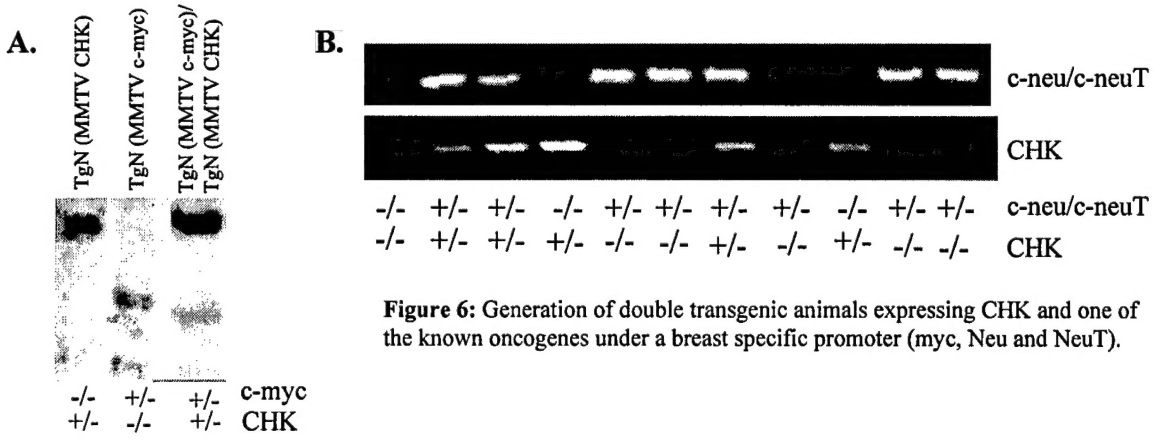

Figure 6: Generation of double transgenic animals expressing CHK and one of the known oncogenes under a breast specific promoter (myc, Neu and NeuT).

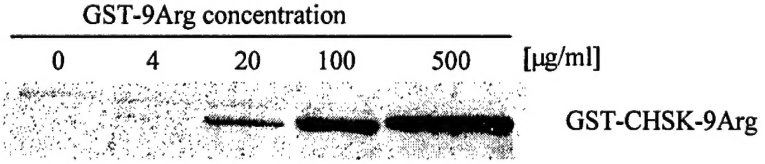

Figure 7: Uptake of GST-9Arg fusion protein from culture medium. Eighty-percent confluent T47D cells were incubated with the various concentrations of GST-9Arg for 30 min. The cells were thoroughly washed in PBS, lysed in protein lysis buffer and then SDS/PAGE plus Western blot analysis was performed with anti-GST antibodies.

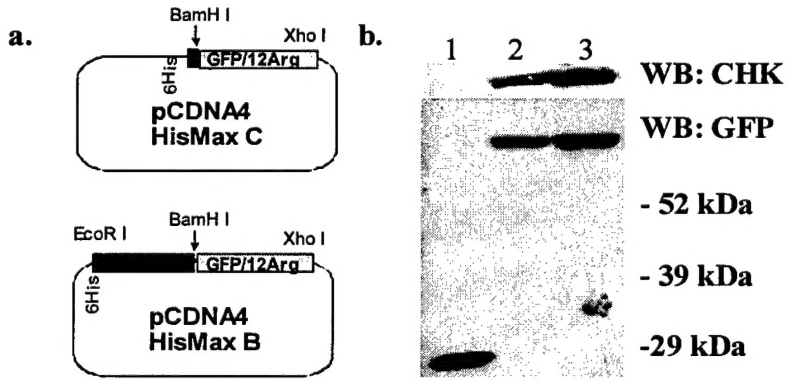

Figure 8: Generation of vectors encoding proteins fused with polyarginine.
a. Maps of mammalian expression vectors generated to express either GFP-12Arg or CHKwt/dk-GFP-12Arg fusion proteins.
b. Western blot analysis of the expression of GFP-12Arg (Lane 1), CHKwt-GFP-12Arg (Lane 2), and CHKdk-GFP-12Arg (Lane 3) proteins in CHO cells.

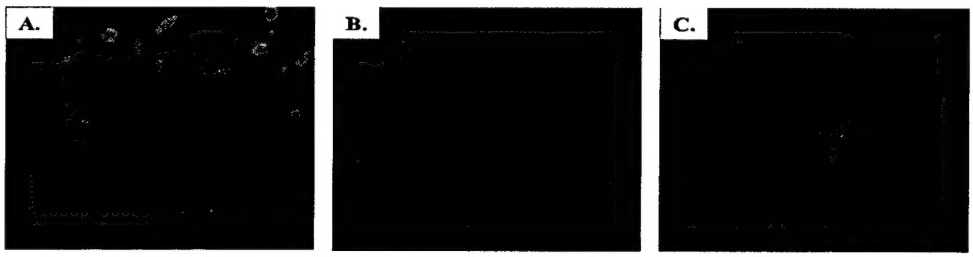

Figure 9: Transient expression of GFP-12Arg and CHK-GFP-12Arg fusion proteins in CHO cells.
The cells were seeded onto a 6-well plate, grown until 80% confluent, and then transfected with 3 μg of GFP-12Arg/pcDNA4HisMaxC (A), CHKdk-GFP-12Arg/pcDNA4HisMaxB (B) or CHKwt-GFP-12Arg/pcDNA4HisMaxB (C) using Lipofectamine 2000 reagent. Fluorescence was assessed 24 h following transfection. Magnification 400x.

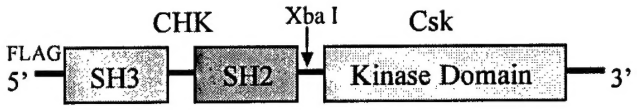

Figure 10: Schematic presentation of CHK/Csk (CHSK) chimeric fusion cDNA.

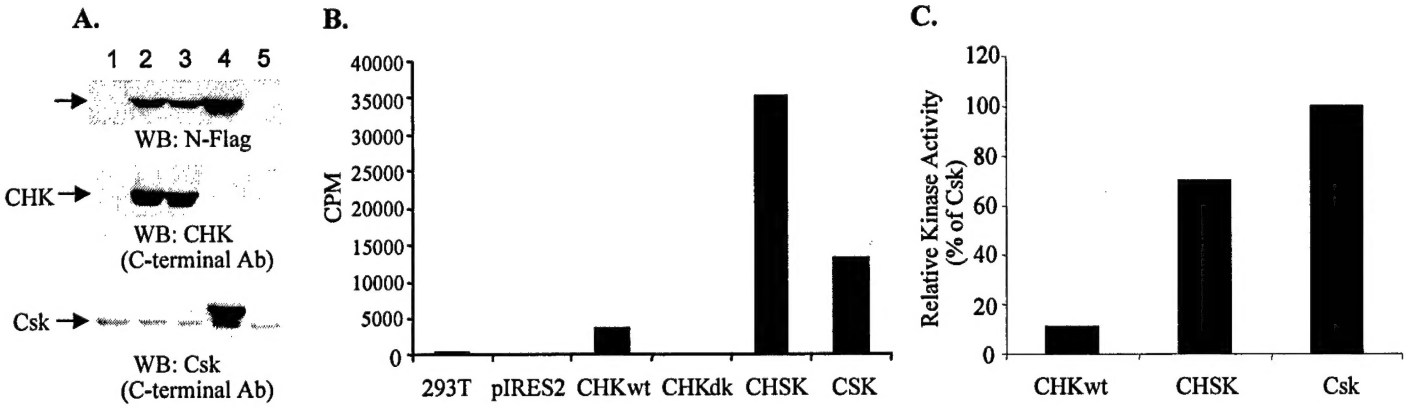

Figure 11: A. Expression of CHK(wt), CHK(dk), and CHSK fusion proteins in 293T cells, as compared to endogenous Csk. 293T cells were seeded onto 6-well plates, grown until 80% confluent, and then untransfected or transfected with 3 μg of empty vector (Lane 1), CHK(wt) (Lane 2), CHK(dk) (Lane 3), CHSK (Lane 4) in pIRES2-EGFP using Lipofectamine 2000 reagent. (Lane 5=untransfected cells). Expression of CHK (wt), CHK(dk), and CHSK as well as endogenous Csk was evaluated by Western blotting using specific antibodies. **B. Tyrosine kinase activity of CHK(wt), CHK(dk), CHSK following transient transformation of 293T cells as compared to endogenous Csk. C. Relative tyrosine kinase activity of CHK(wt) versus CHSK as a percentage of Csk kinase activity.** Cpm reading was normalized for densitometry analysis (Scion Image for Windows software, NIH).